# Hydrodynamic Gene Delivery of Interleukin-22 Protects the Mouse Liver from Concanavalin A-, Carbon Tetrachloride-, and Fas Ligand-Induced Injury *via* Activation of STAT3

**Hongna Pan[1], Feng Hong[1], Svetlana Radaeva[1] and Bin Gao[1, 2]**

**Interleukin-22 (IL-22) is a recently identified T cell-derived cytokine whose biological significance remains obscure. Previously, we have shown that IL-22 plays a protective role in T cell-mediated hepatitis induced by Concanavalin A (Con A), acting as a survival factor for hepatocytes. In the present paper, we demonstrate that hydrodynamic gene delivery of IL-22 cDNA driven either by a liver-specific albumin promoter or a human cytomegalovirus (CMV) promoter results in IL-22 protein expression, STAT3 activation, and expression of several anti-apoptotic proteins, including Bcl-xL, Bcl-2, and Mcl-1 in the liver. Immunohistochemical analysis reveals that IL-22 protein expression is mainly detected in the cytoplasm of hepatocytes. Overexpression of IL-22 by hydrodynamic gene delivery significantly protects against liver injury, necrosis, and apoptosis induced by administration of Con A, carbon tetrachloride ($CCl_4$), or the Fas agonist Jo-2 mAb. Western blot analyses show that overexpression of IL-22 significantly enhances activation of STAT3 and expression of Bcl-xL, Bcl-2, and Mcl-1 proteins in liver injury induced by Con A. In conclusion, hydrodynamic gene delivery of IL-22 protects against liver injury induced by a variety of toxins, suggesting the therapeutic potential of IL-22 in treating human liver disease. *Cellular & Molecular Immunology*. 2004;1(1):43-49.**

**Key Words:** IL-22, STAT3, hydrodynamic gene therapy, liver injury

## Introduction

Interleukin-22 (IL-22) was originally described as an interleukin-10-related T-cell-derived inducible factor (IL-TIF) (1-3). Sharing 22% identity with IL-10, IL-22 belongs to the IL-10 family of cytokines (4-6). Currently, it is believed that IL-22 functions through binding a receptor complex composed of two chains: IL-10Rβ (CRF24, IL-10R2) and IL-22R (CRF2-9) (3-6). The IL-10Rβ subunit has been shown to be a functional component of the IL-10 signaling complex, and is expressed in a wide variety of tissues and cell types (7). In contrast, IL-22R is mainly expressed in the pancreas, small intestine, colon, kidney, and liver, and only in certain cell types (3, 8). Upon binding to the IL-22R and IL-10R2 receptor complex in the liver, IL-22 activates both the Janus kinase-signal transducers and activators of transcription factor (JAK-STAT) and mitogen-activated protein kinase (MAPK) pathways (9). IL-22 has been shown to upregulate expression of acute-phase proteins in the liver (2) and pancreatitis-associated proteins (PAP1) in pancreatic acinar cells (10), suggesting that IL-22 plays a role in inflammatory responses. We have previously demonstrated that IL-22 is significantly elevated in T cell-mediated hepatitis induced by injection of Concanavalin A (Con A) and protects the liver from T cell-induced injury, and *in vitro* experiments reveal that IL-22 is a survival factor for hepatocytes (11). In the present paper, we further demonstrate that hydrodynamic gene delivery of IL-22 protects the liver from Con A-, carbon tetrachloride ($CCl_4$)-, and Fas agonist-induced injury through induction of anti-apoptotic signal (STAT3) and proteins (Bcl-xL, Bcl-2, and Mcl-1).

## Materials and Methods

*Materials*

Anti-STAT3, anti-phospho-STAT3 ($Tyr^{705}$), anti-STAT1, anti-phospho-STAT1 ($Tyr^{701}$) antibodies were obtained from Cell Signaling (Beverly, MA, USA). Other antibodies used in this paper included Mcl-1 and Bcl-2 antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), Bcl-xL antibody (BD PharMingen, San Diego, CA, USA), and anti-IL-22 antibody (R&D System, Minneapolis, MN, USA).

*Construction of pAlb-IL-22 and pCMV-IL-22 expression vectors*

The recombinant murine IL-22 expression vector (pCMV-

[1]Section on Liver Biology, Laboratory of Physiologic Studies, National Institute on Alcohol Abuse and Alcoholism, National Institutes of Health, Bethesda, MD 20892, USA.

[2]Corresponding to: Dr. Bin Gao, Chief, Section on Liver Biology, NIAAA/NIH, Park Bldg Rm 120, 12420 Parklawn Drive, MSC 8115, Bethesda, MD 20892, USA. Tel: 301-443-3998; E-mail: bgao@mail.nih.gov.

*Abbreviation:* IL-22, interleukin-22; JAK, Janus kinase; STAT, signal transducers and activators of transcription factor; Con A, Concanavalin A; $CCl_4$, carbon tetrachloride.

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IL-22) containing the full-length murine IL-22 cDNA driven under a human cytomegalovirus (CMV) promoter was constructed as described previously (11). The recombinant murine IL-22 expression vector (pAlb-IL-22) containing the full-length murine IL-22 cDNA driven under a liver-specific albumin promoter was cloned by inserting murine IL-22 cDNA into the pGEMAlb-SVPA vector. The murine IL-22 cDNA was enzymatically cut from the pCMV-IL-22 vector (11) and then treated with T4 DNA polymerase to generate blunt ends. This IL-22 cDNA fragment was subsequently cloned into the blunt-ended pGEMAlb-SVPA plasmid. The correct orientation was confirmed by appropriate restriction enzyme digestion and sequencing.

*Hydrodynamic plasmid injection*
Plasmid DNA was introduced into murine livers using a hydrodynamic-based gene transfer technique *via* rapid injection of a large volume of DNA solution through the tail vein (12, 13). Hydrodynamic injection is a simple and effective method of transfecting liver cells in mice and rats (12, 13). Briefly, 100μg/mouse DNA was diluted in 2.0 to 2.5ml of saline (0.1ml/g body weight) and injected into the tail vein using a 27-gauge needle and syringe within a time period of 5 to 10 sec. Typically, the mice recovered from the injection within 5 to10 min.

*Murine models of liver injury*
Eight- to ten-week old C57BL/6N mice were purchased from the National Cancer Institute, National Institutes of Health (Frederick, MD, USA). After 2 to 3 days post injection of DNA, mice were administered by Con A (12μg/g body weight) (Sigma, St. Louis, MO, USA) intravenously, or $CCl_4$ (0.1-0.3μg/g body weight) (Sigma) by gavage, or injected with the Fas agonist, Jo-2 mAb (0.2μg/g body weight) (PharMingen) intraperitoneally. The mice were then sacrificed, and the serum was collected for determination of ALT and AST levels at various time points post injection. The livers were also collected for H&E staining.

*Cell extraction, SDS-PAGE and Western blotting*
Cells were lysed using lysis buffer (30mM Tris, pH7.5, 150mM sodium chloride, 1mM phenylmethylsulfonyl fluoride, 1mM sodium orthovanadate, 1% Nonidet P-40, 10% glycerol) for 15 min at 4°C, vortexed and centrifuged at 16,000 rpm at 4°C for 10 min. The supernatants were mixed in Laemmli loading buffer, boiled for 4 min, and then subjected to SDS-PAGE. After electrophoresis, proteins were transferred onto nitrocellulose membranes and blotted against primary antibodies for 16h. Membranes were washed with TPBS (0.05% [vol/vol] Tween-20 in phosphate-buffered saline [pH7.4]) and incubated with a 1:4000 dilution of horseradish peroxidase-conjugated secondary antibodies for 45 min. Protein bands were visualized by an enhanced chemiluminescence reaction (Amersham Pharmacia Biotech, Piscataway, NJ).

*Immunohistochemistry*
Formalin-fixed, paraffin-embedded liver tissue sections were de-paraffinized and re-hydrated with phosphate-buffered saline (PBS), followed by Proteinase K treatment (30μg/ml in 100mmol/L Tris-HCl buffer, 50mmol/L EDTA [pH8.0]) for 30 min at 37°C. To block endogenous peroxidase activity, liver sections were incubated in 0.3% $H_2O_2$ in methanol for 30 min. Nonspecific binding sites were blocked by incubation of the slides with normal blocking serum at 25°C for 20 min. Sections were then incubated with 1:50 diluted primary antibodies, including anti-IL-22 and anti-phospho-STAT3 ($Tyr^{705}$), overnight at 4°C. Biotinylated secondary antibodies and ABC reagent were applied according to the manufacturer's instructions (VECTASTAIN ABC kit, Vector Laboratories, Burlingame, CA). Color development was induced by incubation for 5 to 10 min with the 3,3′-diaminobenzidine (DAB) substrate. Specific staining was visualized by light microscopy.

*Hematoxylin-eosin staining of liver sections*
Following fixation of the livers with 10% formalin/PBS, livers were sliced and stained with hematoxylin-eosin.

*Analysis of alanine transaminase (ALT) and asparate aminotransaminase (AST) activity*
Liver injury was quantified by measuring plasma enzyme activities of ALT and AST using a kit from DREW Scientific (Cumbria, UK).

*Statistical analysis*
Statistical analysis was performed using the Student's test. All $p$ values were two-tailed, and $p<0.05$ was taken as statistically significant.

## Results

*Hydrodynamic gene delivery of IL-22 cDNA results in IL-22 protein expression, STAT3 activation, and expression of several anti-apoptotic proteins*
Two different IL-22 expression vectors were delivered into mice *via* hydrodynamic injection. As shown in Figure 1A, hydrodynamic gene delivery of the IL-22 expression vector under the control of liver-specific albumin promoter (pAlb-IL-22) caused prolonged IL-22 protein expression and STAT3 phosphorylation in the liver, whereas STAT1 was not significantly activated. The peak effect of IL-22 protein expression occurred on day 3 post injection and peak effect of STAT3 activation occurred on day 2 post injection. Expression of anti-apoptotic Bcl-xL protein was also significantly enhanced after hydrodynamic gene delivery of the IL-22 expression vector, with peak effect occurring between day 1 and 3. Expression of anti-apoptotic Bcl-2 and Mcl-1 was also elevated, but was less evident.

Next, the effects of 2 different IL-22 expression vectors controlled by either a liver specific albumin promoter (pAlb-IL-22) or a cytomegalovirus promoter (pCMV-IL-22) were compared. As shown in Figure 1B, injection of the pCMV-IL-22 expression vector induced a 100-fold stronger expression of IL-22 in the liver than injection of the pAlb-IL-22 expression vector (note: comparison was made between lanes loaded with 50μg of proteins in the pCMV-IL-22 group and 150μg of protein in the pAlb-IL-22 and pAlb-vector groups). However, induction of IL-22 protein expression by pCMV-IL-22 injection was transient. As shown in Figure 1B, peak IL-22 protein expression occurred on day 1 post injection and rapidly declined after day 2. In contrast, the peak effect of IL-22 protein expression occurred 3 days post injection of pAlb-IL-22

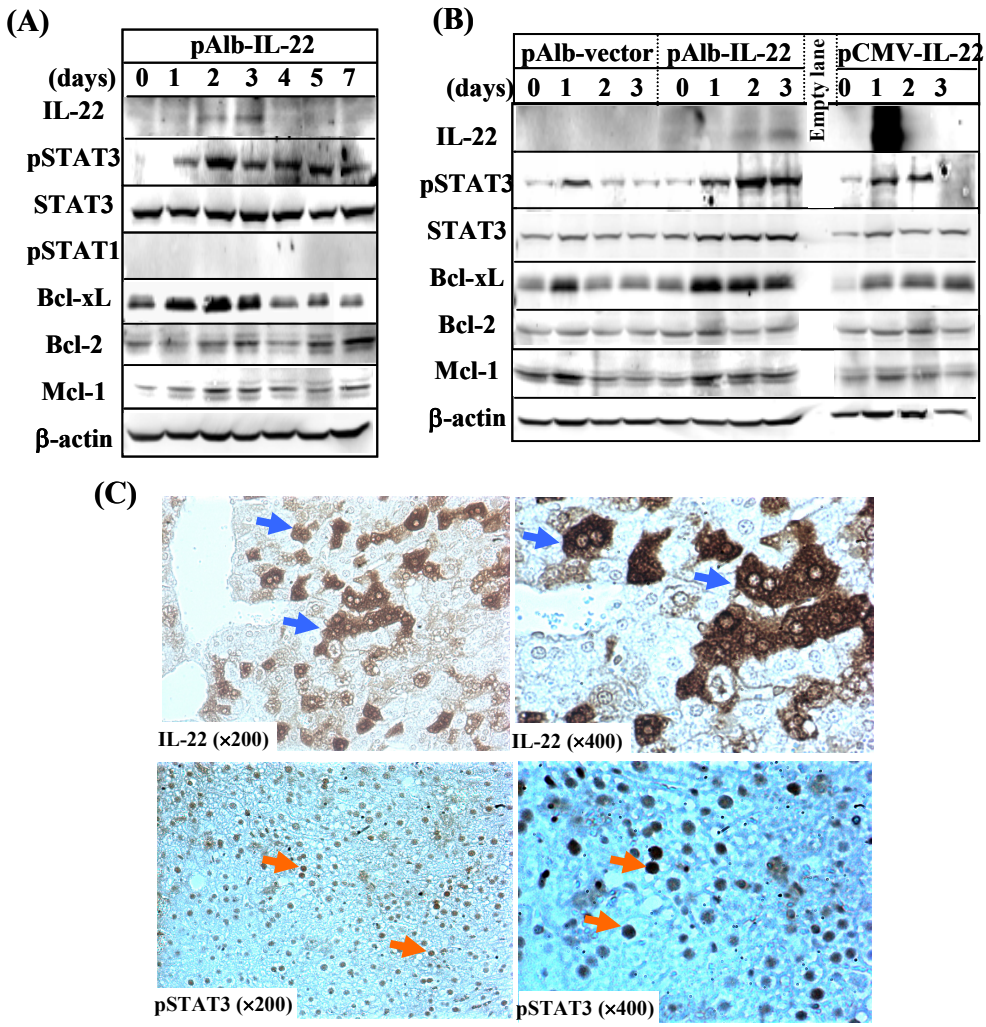


**Figure 1.** Hydrodynamic gene delivery of IL-22 cDNA results in IL-22 protein expression, STAT3 activation, and expression of several anti-apopotic proteins in the liver. (A) (B) C57BL/6N mice (7-8 weeks old) were rapidly injected in the tail vein with pAlb-IL-22 DNA (100μg/mouse) (panel A), or with pAlb-vector DNA, pAlb-IL-22 DNA, and pCMV-IL-22 DNA (100μg/mouse) (panel B). Mice were sacrificed, and livers were collected and homogenized on various days post injection. Liver extracts were prepared and subjected to Western blot analysis with various antibodies as indicated. In panel A, each lane was loaded with 150μg protein extracts. In panel B, 150 μg protein extracts from pAlb-vector and pAlb-IL-22 groups were loaded into lanes, and 50μg protein extracts from the pCMV-IL-22 group were loaded into lanes. (C) Photomicrographs of representative IL-22 and phosphorylated STAT3 immunostaining in liver tissues after 1 day of pCMV-IL-22 injection were shown (magnifications are shown in the panels). About 30% to 40% of hepatocytes were stained positively with the anti-IL-22 antibody, and about 95% of hepatocytes were stained positively with the anti-phospho-STAT3 ($Tyr^{705}$) antibody. Blue arrows indicate IL-22 protein in the cytoplasm of hepatocytes and red arrows indicate phosphorylated STAT3 in the nuclei of hepatocytes.

(Figure 1A). Hydrodynamic delivery of pCMV-IL-22 also induced significant activation of STAT3, Bcl-xL, Bcl-2, and Mcl proteins. Although induction of IL-22 protein expression was much greater in pCMV-IL-22-treated mice, the activation of STAT3 and Bcl-xL was not significantly greater compared to pAlb-IL-22-treated mice. This may be due to prolonged IL-22 protein expression in pAlb-IL-22-treated mice compared to the transient expression of IL-22 protein in pCMV-IL-22-treated mice.

Immunohistochemical analysis showed that about 30% to 40% of hepatocytes expressed IL-22 protein 1 day after injection of pCMV-IL-22, and that IL-22 protein was found mainly in the cytoplasm of hepatocytes (Figure 1C). Phosphorylated STAT3 was detected in the nuclei of about 95% of hepatocytes.

*Hydrodynamic gene delivery of IL-22 cDNA protects mice from Con A-induced liver injury*

We have previously shown that IL-22 blockaded with a neutralizing antibody worsens Con A-induced liver injury, whereas injection of recombinant IL-22 protein attenuates such injury (11). To further confirm the protective role of IL-22 in this model, we examined the effects of hydrodynamic gene delivery of IL-22 cDNA on liver injury induced by Con A. Hydrodynamic delivery of DNA was shown to cause transient liver damage (13). Here we also demonstrated that ALT levels peaked 24h after injection around 1382±482U/L, and significantly declined on day 2, and returned to normal levels on day 3 (Figure 2A). Since hepatic IL-22 protein expression peaked on day 3 post pAlb-IL-22 injection (Figure 1A) and transient liver

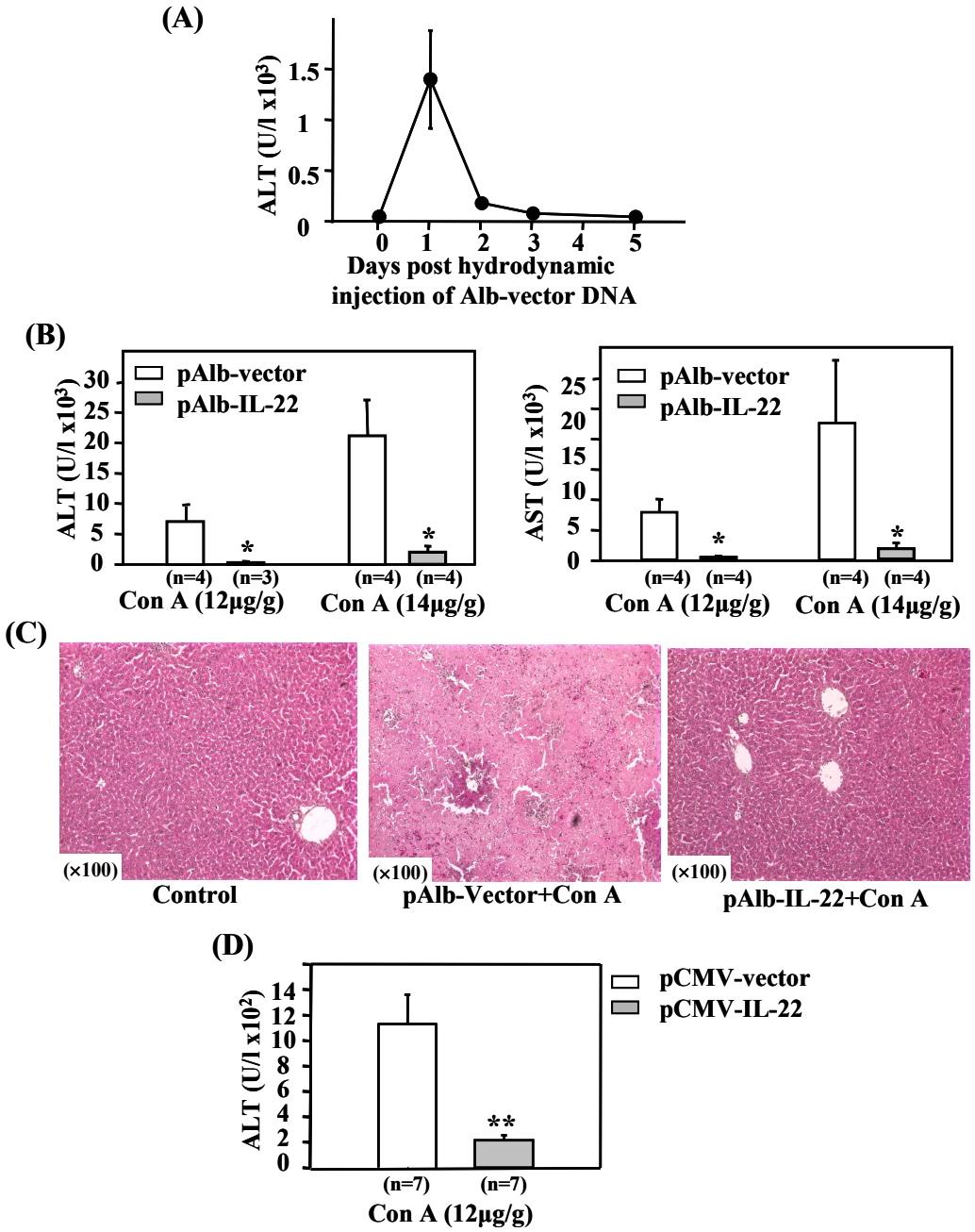


**Figure 2.** Hydrodynamic gene delivery of IL-22 cDNA protects mice from Con A-induced liver injury. (A) Hydrodynamic injection caused transient liver damage. C57BL/6N mice (7-8 weeks old) were rapidly injected in the tail vein with pAlb-vector (100μg/mouse). After various days, mice were sacrificed, and serum was collected for determination of ALT levels. (B) (C) C57BL/6N mice (7-8 weeks old) were rapidly injected in the tail vein with pAlb-vector DNA (100μg/mouse) or pAlb-IL-22 cDNA (100μg/mouse), followed 3 days later by intravenous injection of Con A. Mice were then killed 9h post Con A injection, and serum was collected for determination of ALT and AST levels (panel B) and livers were collected for H&E staining (panel C). (D) C57BL/6N mice (7-8 weeks old) were rapidly injected in the tail vein with pCMV-vector DNA (100μg/mouse) or pCMV-IL-22 DNA (100μg/mouse), followed 2 days later by intravenous injection of Con A. Mice were then killed 9h post Con A injection, and serum was collected for determination of ALT levels. Values in panels B and D represent means±SD from 3 to 7 mice in each group as indicated. * $p$ <0.05, ** $p$ <0.01 *vs* the corresponding control group (white bars).

damage caused by hydrodynamic injection was recovered at this time point, we chose this time point to inject Con A. As shown in Figure 2B, administration of 12μg/g and 14μg/g of Con A caused significant elevations in serum ALT and AST levels 9h post injection in mice treated with the pAlb-vector DNA, which were markedly suppressed in mice treated with the pAlb-IL-22. Liver histology showed that Con A injection caused massive necrosis in the livers of pAlb-vector-treated mice, but only caused spotted necrosis in the livers of pAlb-IL-22-treated mice (Figure 2C). Finally, hydrodynamic injection of pCMV-IL-22 also significantly protected the mouse livers from Con A-induced elevation of serum ALT levels (liver injury) (Figure 2D, ** $p$<0.01).

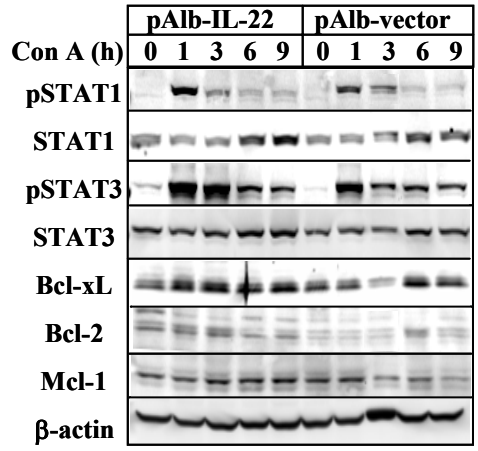


**Figure 3.** Injection of Con A causes higher hepatic STAT3 activation and Bcl-xL expression in pAlb-IL-22 cDNA-treated mice compared to pAlb-vector DNA-treated mice. C57BL/6N mice (7-8 weeks old) were rapidly injected in the tail vein with pAlb-vector DNA (100μg/mouse) or pAlb-IL-22 DNA (100μg/mouse), followed 3 days later by intravenous injection of Con A. Mice were then killed 1 to 9h post Con A injection, and livers were collected for Western blot analyses using various antibodies as indicated. Data are representative of 3 independent experiments with similar results. P denotes the phosphorylated form.

*Con A injection causes higher hepatic STAT3 activation and Bcl-xL expression in pAlb-IL-22-treated mice compared to pAlb-vector-treated mice*

To understand the underlying mechanism by which hydrodynamic gene delivery of IL-22 cDNA protects mice from Con A-induced liver injury, we compared activation of STAT1 and STAT3 in both pAlb-vector- and pAlb-IL-22-treated groups. As shown in Figure 3, Con A injection rapidly activated both STAT1 and STAT3. Activation of STAT1 was slightly enhanced in pAlb-IL-22-treated mice compared to pAlb-vector-treated mice 1h post Con A administration. Consistent with this, induction of STAT1 protein expression, which is controlled by IFN-γ/STAT1 (14), was also slightly higher in the pAlb-IL-22-injected group than in the pAlb-vector-injected group. Hepatic STAT3 activation was significantly higher in pAlb-IL-22-treated group compared to pAlb-vector-treated mice 1 and 3 h after administration of Con A, whereas expression of STAT3 protein remained unchanged. It was also noted that a significantly higher basal level of phosphorylated STAT3 was detected in the livers of pAlb-IL-22-treated group compared to the pAlb-vector-treated group (Con A-treated 0 time point). Consistent with this finding, Con A-induced expression of Bcl-xL, which is controlled by STAT3 (14), was significantly higher in pAlb-IL-22-treated mice than in pAlb-vector-treated mice. Expression of Bcl-2 and Mcl-1 was slightly elevated after administration of Con A, which was more evident in pAlb-IL-22-injected mice compared to pAlb-vector-treated mice.

*Hydrodynamic gene delivery of pAlb-IL-22 protects mice from $CCl_4$-induced liver injury*

The protective effect of IL-22 was also tested in another murine model of liver injury induced by injection of $CCl_4$.

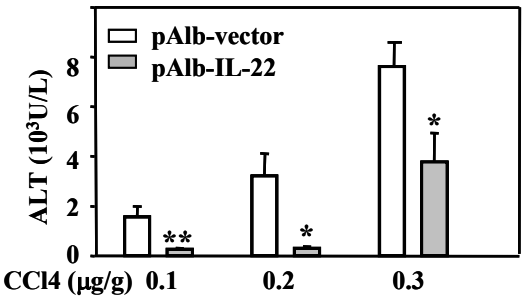


**Figure 4.** Hydrodynamic gene delivery of pAlb-IL-22 cDNA protects mice from $CCl_4$-induced liver injury. C57BL/6N mice (7-8 weeks old) were rapidly injected in the tail vein with pAlb-vector DNA (100μg/mouse) or pAlb-IL-22 cDNA (100μg/mouse), followed 3 days later with $CCl_4$ (0.1-0.3μg/g body weight) administration by oral gavage. Mice were then killed 24h post administration of $CCl_4$, and the serum was collected for determination of ALT levels. Values represent means±SD from 3 to 4 mice in each group as indicated. *$p$<0.05, **$p$<0.01 *vs* the corresponding control group (white bars).

As shown in Figure 4, administration of $CCl_4$ caused liver injury (elevation of serum ALT levels) dose-dependently in pAlb-vector-treated mice. Such injury was markedly suppressed in mice treated with pAlb-IL-22 (*$p$<0.05 and **$p$<0.01).

*Hydrodynamic gene delivery of IL-22 protects mice from Fas agonist-induced liver injury and apoptosis*

The above data showed that hydrodynamic gene delivery of IL-22 cDNA protected mice from Con A- and $CCl_4$-induced liver injury. However, liver injury caused by Con A or $CCl_4$ results from the interaction of a wide variety of cells, cytokines, and direct cytotoxicity. To examine whether the hepatoprotective effect of IL-22 is mediated by directly protecting against hepatocyte death, we used a murine model of liver injury induced by injection of the Fas agonist, Jo-2 mAb. As shown in Figure 5A and 5B, injection of Jo-2 mAb rapidly induced serum ALT elevation, which was markedly suppressed in mice receiving hydrodynamic gene delivery of both pAlb-IL-22 and pCMV-IL-22 expression vectors. The protective effect of pCMV-IL-22 against Jo-2 mAb-induced liver injury was more evident than that of pAlb-IL-22. As shown in Figure 5, serum ALT level 6h post Jo-2 mAb injection was reduced by 50% in mice treated with pAlb-IL-22 (Figure 5A), but reduced by 80% in mice treated with pCMV-IL-22 (Figure 5B). Histologic examination showed that injection of Jo-2 mAb caused massive apoptotic injury, congestion, and parenchymal collapse in the livers of mice receiving pCMV-vector treatment, which were significantly alleviated in mice receiving pCMV-IL-22 DNA treatment (Figure 5C).

## Discussion

IL-22 was originally identified by cDNA subtraction as a gene specifically induced by IL-9 in mouse T cells and was subsequently found in human cells in 2000 (1-3). The receptor complex binding IL-22 was also identified, comprised of the IL-10Rβ (CRF2-4, IL-10R2) and IL-22R

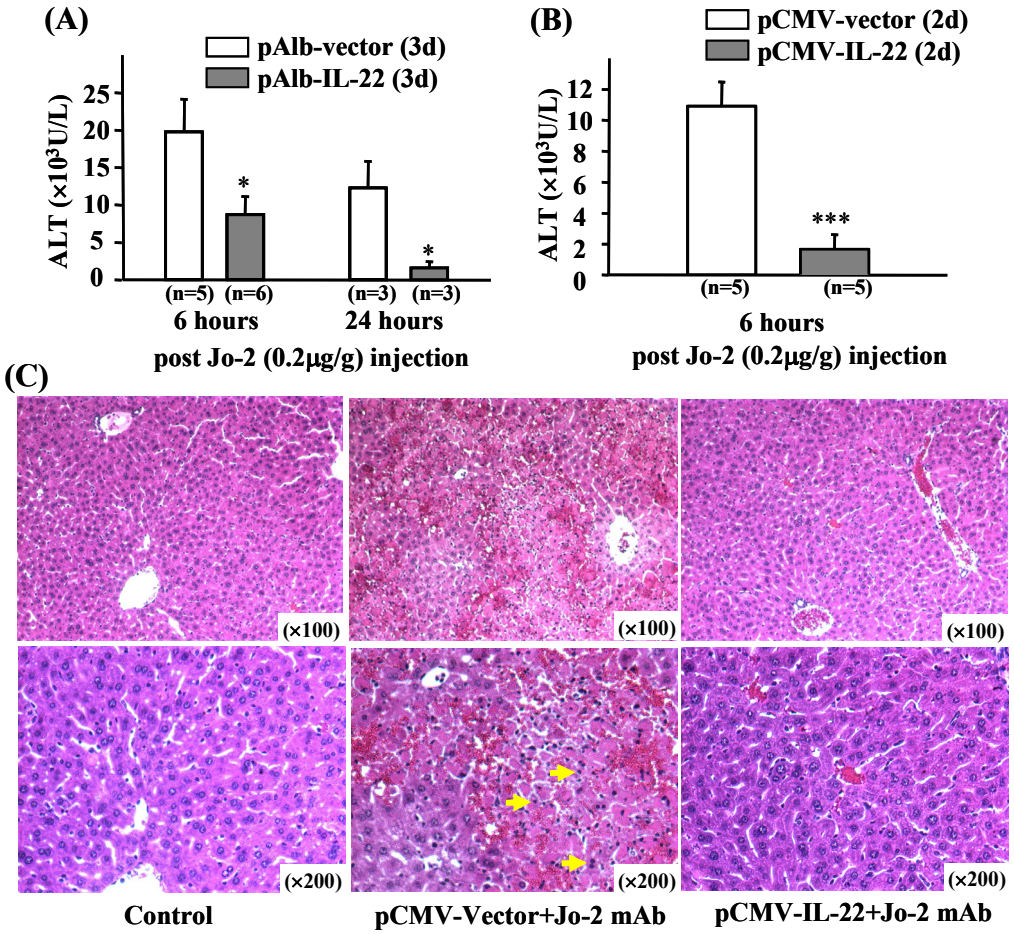


**Figure 5.** Hydrodynamic gene delivery of IL-22 cDNA protects mice from Fas agonist (Jo-2 mAb)-induced liver injury and apoptosis. (A)(B) C57BL/6N mice (7-8 weeks old) were rapidly injected in the tail vein with pAlb-vector DNA (100μg/mouse) or pAlb-IL-22 cDNA (100μg/mouse), or with pCMV-vector DNA (100μg/mouse) or pCMV-IL-22 cDNA (100μg/mouse) (panel B), followed 3 days (panel A) or 2 days (panel B) later with intraperitoneal administration of the Fas agonist, Jo-2 mAb (0.2μg/g body weight). Mice were then killed 6h and 24h post administration of Jo-2 mAb, and the serum collected for determination of ALT levels. Values represent means±SD from 3 to 6 mice in each group as indicated. *$p<0.05$, ***$p<0.001$ *vs* the corresponding control group (white bars). (C) Liver tissues from the mice in panel B were fixed and subjected to H&E staining (original magnification, ×100 and ×200). Control represents H&E staining of normal C57BL/6N mouse livers. Note: massive necrosis, apoptosis, and hemorrhage in the lives of pCMV-vector+Jo-2 mAb group. Yellow arrows indicate apoptotic hepatocytes.

(CRF2-9) subunits (3). However, little is known about the biological significance of IL-22. Previously, we provided evidence suggesting that IL-22, produced by activated T cells, acts as a protective cytokine to attenuate liver injury in T cell-mediated hepatitis and that IL-22 acts as a survival factor for hepatocytes (11). The protective role of IL-22 in T cell-induced hepatitis is likely mediated *via* activation of STAT3 and the subsequent induction of anti-apoptotic proteins such as Bcl-xL, Bcl-2 and Mcl (11). Here, we further confirmed the hepatoprotective function of IL-22 in T cell-mediated hepatitis *via* hydrodynamic gene delivery. As shown in Figure 2, hydrodynamic gene delivery of 2 different IL-22 expression vectors markedly prevented Con A-induced liver necrosis and injury. The hepatoprotective function of IL-22 was also tested in 2 other models of liver injury induced by $CCl_4$ and a Fas agonist, and our results showed that hydrodynamic gene delivery of IL-22 protects against liver injury in both models (Figure 4 and 5). Since the Fas agonist Jo-2 mAb-induced liver injury results from direct activation of the apoptotic cell death program in hepatocytes (15), our findings that IL-22 protected the mouse liver from Jo-2 mAb-induced liver injury suggests that IL-22 directly protects hepatocytes from injury *in vivo*, which may be an important mechanism contributing to the hepatoprotective functions of IL-22 *in vivo*. Activation of STAT3 has previously been implicated in the ability of IL-6 to protect against various forms of liver injury (14, 16-23) *via* induction of several anti-apoptotic proteins, including Bcl-xL, Bcl-2, and Mcl-1 (24-28). Previously, we showed that IL-22 activated STAT3 in the liver *in vivo* and in hepatocytes *in vitro*, and subsequently induced Bcl-xL, Bcl-2, and Mcl-1 in hepatocytes (11). In the present paper, we further show that hydrodynamic gene delivery of IL-22 markedly activates STAT3 and enhances expression of Bcl-xL, Bcl-2, and Mcl-1 proteins in the liver *in vivo*. Taken those together, our findings suggest that the hepato-protective function of IL-22 *in vivo* is mediated *via* STAT3 activation and consequently, the induction of anti-apoptotic

proteins.

The hepatoprotective functions of IL-6 has been well documented in a variety of models of liver injury (14, 16-20), and IL-6 has been proposed as a therapeutic agent to prevent fatty liver transplantation failure (20, 29), partial liver transplantation failure (30), ischemia/reperfusion liver injury (31), acute liver failure (32, 33) and liver fibrosis (16, 22). However, the *in vivo* application of IL-6 in humans has been hampered due to significant clinical side effects (34). Since IL-22 activates similar anti-apoptotic signals and proteins as IL-6 in the liver, it is likely that IL-22 also has broad hepatoprotective functions as IL-6 but with less systemic side effects, for IL-22R is expressed only in restricted organs (such as the pancreas, kidney, liver, and lung) whereas the IL-6 receptor complex (gp80 and gp130) is expressed ubiquitously. Therefore, the therapeutic application of the hepatoprotective cytokine IL-22 in treating human liver disease should be further explored.

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